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Purification and Characterization of Heparin-binding Endothelial Cell Growth Factors*

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Roy Lobb‡, Joachim Sasse§, Robert Sullivan§, Yuen Shing§, Patricia D'Amore¶, Jeffrey Jacobs§, and Michael Klagsbrun§||

From the ‡Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, and Brigham and Women's Hospital and the Departments of §Surgery, ¶Pathology, and ||Biological Chemistry, Harvard Medical School, and The Children's Hospital, Boston, Massachusetts 02115

Thirteen endothelial cell growth factors have been purified to homogeneity by heparin affinity and reversed-phase high performance liquid chromatography, and their chromatographic and electrophoretic properties were compared. The amino acid compositions of 10 of these mitogens have also been determined. The results indicate that these heparin-binding growth factors (HBGFs) can be subdivided into two classes. Class 1 HBGFs are anionic mitogens of molecular weight 15,000–17,000 found in high levels in neural tissue and include acidic brain fibroblast growth factor and retina-derived growth factor. Class 2 HBGFs are cationic mitogens of molecular weight 18,000–20,000 found in a variety of normal tissues and are typified by pituitary fibroblast growth factor and cartilage-derived growth factor. Typical class 2 HBGFs have also been isolated from a rat chondrosarcoma, a human melanoma, and a human hepatoma, suggesting that tumors do not make a structurally distinct HBGF class. These results provide a sound basis for the evaluation of the HBGFs purified from a variety of tissues and species and for the delineation of their normal and pathological functions *in vivo*.

Endothelial cell growth factors have been isolated from a variety of sources including pituitary (1), brain (2–5), hypothalamus (6, 7), retina (8), and cartilage (9). We discovered that a tumor-derived endothelial cell growth had a strong affinity for heparin and that the use of heparin affinity chromatography allowed its purification to homogeneity in only two steps (10, 11). Subsequently, we and other workers have found that many if not all endothelial cell growth factors have a strong affinity for heparin (12–22), and heparin affinity chromatography has been used to purify to homogeneity growth factors found in brain, hypothalamus, cartilage, and tumors (11, 14, 16, 18, 19, 21).

Although mitogens from different sources differ in apparent molecular weight (ranging from 16,000 to 20,000) and in isoelectric point (ranging from 5 to 10), their common property of heparin affinity suggested that they might be structurally related. To explore this possibility, we have purified to

homogeneity 13 endothelial cell growth factors from a variety of sources and tissues and have compared a number of their biochemical properties. These properties include: (i) chromatographic behavior on heparin-Sepharose, (ii) isoelectric point, (iii) chromatographic behavior on C3 reversed-phase HPLC¹ columns, (iv) electrophoretic behavior on SDS-PAGE, and (v) amino acid composition. The results demonstrate that all polypeptide endothelial cell growth factors described so far fall into two classes. Within each class they are structurally very closely related and in some cases may be identical.

EXPERIMENTAL PROCEDURES

Growth Factor Isolation—The heparin-binding mitogens present in bovine brain and hypothalamus were purified to homogeneity as described previously (14). Those in human brain were purified by a minor modification of this procedure (21). The purification of bovine, human, and chick cartilage-derived growth factor, retina-derived growth factor, and rat chondrosarcoma growth factor followed published procedures (11, 15, 16). A human hepatoma-derived mitogen was purified from lysates of SK-HEP cells by Bio-Rex 70 cation-exchange and heparin-Sepharose affinity chromatography using methods as described for the rat chondrosarcoma mitogen (11). A human malignant melanoma-derived mitogen was purified from extracts of solid tumors by repeated cycling over heparin-Sepharose essentially as described for cartilage-derived growth factor (16). The melanoma line (Mewo) was provided by Dr. Jorgen Fogh (Human Tumor Cell Bank, Sloan-Kettering Institute, New York) and grown in nude rats (Taconic Farms, Inc., Germantown, NY).

High Performance Liquid Chromatography—Reversed-phase HPLC was performed on a Waters Associates liquid chromatography system consisting of an LKB 2138 206 nm detector, two model 6000 solvent delivery systems, and a propylsilane (C3) column (Ultrapore RSPC, 5- μ m particle size, 75 \times 4.6 mm, Beckman Instruments). Peaks of growth factor activity obtained from the heparin-Sepharose columns were applied directly to the C3 column via one pump. The column was washed with 0.1% (v/v) trifluoroacetic acid in water until absorption returned to base line, and the mitogens were eluted from the column with a linear gradient of 0–60% acetonitrile, 0.1% trifluoroacetic acid in water. Elution was performed over 30 min at a flow rate of 0.8 ml/min. One-minute (0.8-ml) fractions were collected into siliconized tubes. Aliquots of the C3 column fractions were diluted at least 40-fold into physiologic saline containing 0.1% bovine serum albumin immediately following completion of the column run to minimize loss of biological activity.

Assays—We have established that heparin-binding mitogens in brain, hypothalamus, pituitary, retina, cartilage, and tumors are potent growth factors for capillary endothelial cells (11, 13–16). We have also found that BALB/c 3T3 cells are highly responsive to these same mitogens and serve as a rapid and reliable target cell for their assay during purification (11, 13–16). In the studies reported here, only 3T3 cell stimulation was used. Stimulation of quiescent, con-

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¹ The abbreviations used are: HPLC, high performance liquid chromatography; HBGF, heparin-binding growth factor; FGF, fibroblast growth factor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

TABLE I
Heparin affinity and isoelectric point of heparin-binding growth factors

Mitogen source	Species	Heparin affinity ^a	Ref.	pI	Ref.
		M			
Brain	Bovine, human	0.9–1.1	13, 14, 21	5.0–5.8	5, 17, 18
Brain	Bovine, human	1.4–1.6	13	9.6	4
Hypothalamus	Bovine	0.9–1.1	13, 14, 15	5.0	13, 15
Hypothalamus	Bovine	1.3–1.5	13	8.0	13
Retina	Bovine	0.9–1.1	15	5.0	15
Cartilage	Bovine, human, chick	1.6–1.8	16	9.8	^b
Chondrosarcoma	Rat	1.3–1.5	11	9.8	^b
Melanoma	Human	1.6–1.8	^b	ND ^c	
Hepatoma	Human	1.6–1.8	^b	ND	

^a Sodium chloride concentration required for elution from heparin-Sepharose with a linear gradient of NaCl in 10 mM Tris-HCl, pH 7.0.

^b M. Klagsbrun, J. Sasse, Y. Shing, and R. Sullivan, unpublished observations.

^c ND, not determined.

fluent monolayers of 3T3 cells was measured in 96-well plates by standard techniques as described elsewhere (14). One unit of activity is defined as the amount of mitogen required to stimulate half-maximal DNA synthesis in the 3T3 cell assay.

Other Techniques—Analytical SDS-PAGE was performed on vertical slab gels according to standard techniques (23). Gels were first silver-stained by the method of Oakley *et al.* (24) and then counterstained using a commercially available kit (Bio-Rad), resulting in greater sensitivity than either method alone. Isoelectric focusing in sucrose gradients was performed as described (13). Amino acid analysis was performed by the "Pico-Tag" method (Waters Associates) (25).

RESULTS

Heparin-Sepharose Chromatography—We have purified to homogeneity 13 heparin-binding growth factors (HBGFs) from the following sources: bovine and human brain; bovine hypothalamus; bovine retina; bovine, human, and chick cartilage; two solid tumors, a rat chondrosarcoma and a human melanoma; and an established human hepatoma cell line. Table I summarizes the chromatographic properties of these mitogens on heparin-Sepharose. Isoelectric points, established by ourselves and others, are also included. The HBGFs fall into two groups on the basis of their differential affinity for heparin. One group of mitogens elutes between 0.9 and 1.1 M NaCl (class 1), and the second between 1.3 and 1.8 M NaCl (class 2). Isoelectric focusing studies suggest that the former are anionic mitogens and the latter cationic mitogens (Table I).

Reversed-phase Chromatography—All of the mitogens listed in Table I were subjected to reversed-phase HPLC on C3 columns and eluted with a linear gradient of acetonitrile. The elution position of each growth factor was determined by bioassay. The two groups of mitogen are distinguishable by their behavior on reversed-phase HPLC. The anionic (class 1) HBGFs from bovine and human brain, bovine hypothalamus, and bovine retina all elute from the C3 column at acetonitrile concentrations of 38–42%. Typical profiles for the anionic HBGFs from human and bovine brain and bovine retina are shown in Fig. 1 (A–C). In contrast, cationic HBGFs are less hydrophobic and elute at acetonitrile concentrations of 29–34%. These include HBGFs from bovine hypothalamus, bovine and human brain, human and chick cartilage, and the three tumor-derived mitogens. Typical profiles for the cationic HBGFs from bovine brain, human cartilage, and the rat chondrosarcoma are shown in Fig. 1 (D–F).

SDS-PAGE—All 13 HBGFs have been analyzed by SDS-PAGE and silver staining. All mitogens were homogeneous by this criterion. Fig. 2 shows eight HBGFs run under identical conditions. Anionic growth factors derived from bovine

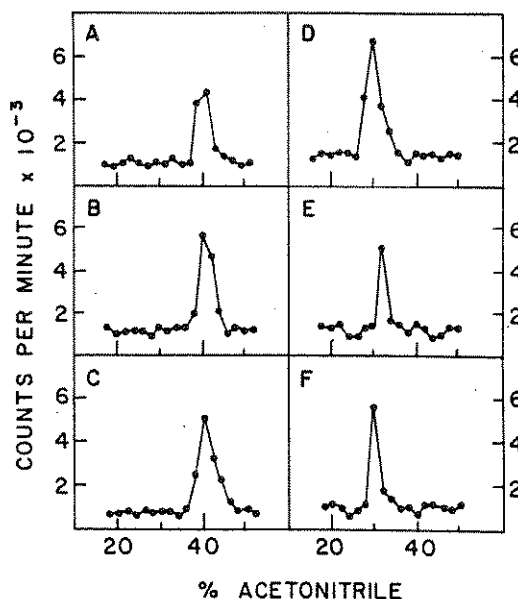


FIG. 1. Reversed-phase HPLC of six HBGFs. Mitogens eluted from heparin-Sepharose were applied directly to a reversed-phase C3 column equilibrated with 0.1% trifluoroacetic acid in water at flow rates of 1.5–2.0 ml/min. The column was washed with 0.1% trifluoroacetic acid in water until absorbance at 206 nm returned to its base-line value and then eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (2.5%/ml, 0.8 ml/min). The bioassay profiles shown are for mitogens from the following sources: (anionic) bovine brain (A), (anionic) human brain (B), bovine retina (C), (cationic) bovine brain (D), human cartilage (E), and rat chondrosarcoma (F).

retina (lane 1) and bovine brain (lane 2) have molecular weights of about 16,000, whereas anionic human brain-derived growth factor (lane 3) has a molecular weight of about 17,000. Cationic growth factors derived from bovine brain (lane 4), human brain (lane 5), and a rat chondrosarcoma (lane 7) have molecular weights of about 18,000. Cationic growth factors derived from human cartilage (lane 6) and a human hepatoma (lane 8) have molecular weights of about 19,000. It appears that anionic class 1 growth factors (lanes 1–3) have lower molecular weights than cationic class 2 growth factors (lanes 4–8). Anionic and cationic bovine hypothalamus-derived growth factors have molecular weights identical to those of anionic and cationic bovine brain-derived mitogens, respectively (Ref. 14 and results not shown). Bovine and chick cartilage-derived growth factors and a human melanoma-derived mitogen have molecular weights of 18,000–19,000 (results not shown).

1 2 3 4 5 6 7 8

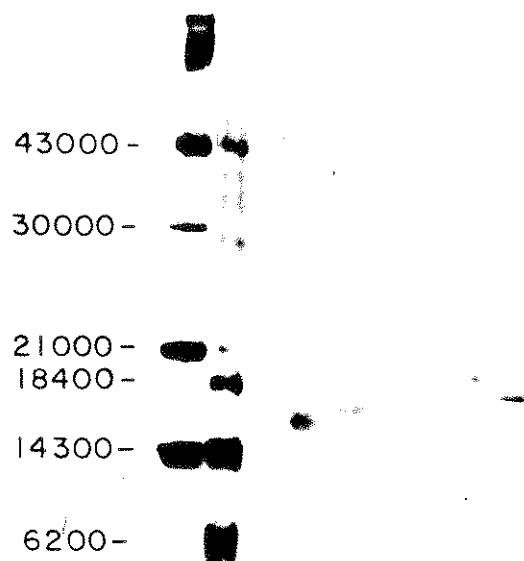


FIG. 2. SDS-PAGE of eight HBGFs. Growth factors were purified by a combination of heparin-Sepharose chromatography and reversed-phase chromatography on C3 columns. The preparations were analyzed by SDS-PAGE and silver staining. The first two lanes contain Bio-Rad and Bethesda Research Laboratories molecular weight markers, respectively. Molecular weights are indicated at the left. Only lanes with growth factor (50–200 ng) are numbered. Sources are bovine retina (lane 1), (anionic) bovine brain (lane 2), (anionic) human brain (lane 3), (cationic) bovine brain (lane 4), (cationic) human brain (lane 5), human cartilage (lane 6), rat chondrosarcoma (lane 7), and human hepatoma (lane 8).

Amino Acid Compositions—Amino acid compositions have been determined for 10 HBGFs. The results (Tables II and III) show unequivocally that HBGFs can be grouped into two classes. Class 1 HBGFs (Table II) include the anionic mitogens found in bovine and human brain, bovine hypothalamus, and bovine retina. The amino acid compositions of the anionic mitogens in bovine brain and hypothalamus are the same as that of acidic bovine brain FGF (5), within experimental error. The mitogen in bovine retina has a very similar amino acid composition, suggesting that it is closely related and possibly identical to those in brain and hypothalamus. The mitogen in human brain is clearly very closely related to those found in bovine neural tissue, suggesting that the structure of anionic class 1 HBGFs is very highly conserved.

Class 2 HBGFs (Table III) include the cationic mitogens found in human cartilage, bovine and human brain, and bovine hypothalamus, as well as mitogens found in two different tumors. The amino acid composition of pituitary FGF (26) demonstrates that this cationic mitogen is also a class 2 HBGF (Table III). This mitogen class also appears to be structurally very closely related, independent of tissue or species.

The amino acid compositions of HBGFs in each class have several distinctive features. The anionic class 1 mitogens have only 1 methionine and a very high leucine content. Indeed, leucine accounts for 12–14% of the molecule. The cationic class 2 mitogens have more methionine than class 1 HBGFs and double the arginine content, but less histidine (3 *versus*

TABLE II

Amino acid compositions of class 1 HBGFs

24-h hydrolysates of 6–20 pmol of proetin are shown (average of four to six independent analyses, except bovine retinal HBGF, which is a single analysis). The nearest integer values are given in parentheses.

Amino acid	Acidic brain FGF ^a	Bovine brain	Bovine hypothalamus ^b	Bovine retina	Human brain
Asx	14	14.7 (15)	15.1 (15)	13.7 (14)	15.3 (15)
Glx	16	17.0 (17)	17.6 (18)	14.5 (15)	17.0 (17)
Ser	10	9.2 (9)	9.6 (10)	7.7 (8)	10.0 (10)
Gly	14	14.3 (14)	16.4 (16)	16.0 (16)	15.2 (15)
His	5	5.4 (5)	5.4 (5)	4.6 (5)	5.0 (5)
Arg	6	6.0 (6)	5.6 (6)	6.8 (7)	6.4 (6)
Thr	9	8.6 (9)	8.8 (9)	10.0 (10)	8.5 (9)
Ala	5	5.4 (5)	5.1 (5)	5.2 (5)	4.7 (5)
Pro	7	7.9 (8)	7.8 (8)	6.6 (7)	8.9 (9)
Tyr	7	6.6 (7)	7.3 (7)	7.2 (7)	7.8 (8)
Val	5	4.9 (5)	4.4 (4)	6.2 (6)	5.0 (5)
Met	1	1.1 (1)	1.3 (1)	1.2 (1)	0.8 (1)
Ile	6	6.0 (6)	5.6 (6)	7.2 (7)	4.4 (4)
Leu	19	18.4 (18)	20.4 (20)	19.2 (19)	18.1 (18)
Phe	7	6.6 (7)	7.0 (7)	7.7 (8)	4.3 (4)
Lys	13	13.0 (13)	12.9 (13)	13.1 (13)	12.2 (12)
Total	144	145	150	148	143

^a From Ref. 5.

^b From Ref. 14.

5) and leucine (12–13 *versus* 18–20). These differences identify unequivocally the HBGF class to which a mitogen belongs.

DISCUSSION

Virtually all of the polypeptide mitogens for endothelial cells that have been described in the last decade can now be purified rapidly and efficiently using heparin affinity chromatography (10–22). Growth factors found in chondrosarcoma tissue (11), brain (14, 18, 19), hypothalamus (14), cartilage (16), and pituitary (19) have been purified to homogeneity by this means. Other endothelial cell growth factors that have been partially purified by heparin affinity chromatography include the endothelial cell growth factor of Maciag *et al.* (17), retina-derived growth factor (15), and mitogens in macrophages (20) and placenta (14, 22). We refer to these endothelial cell growth factors as heparin-binding growth factors because: (i) heparin binding is their only common biochemical property, (ii) heparin affinity chromatography has become an essential step for their efficient purification, and (iii) heparin interacts with some of these growth factors to enhance their mitogenic activity *in vitro* (27, 28) and their angiogenic activity *in vivo* (29).

The structural relationship between the many endothelial cell growth factors that have been reported has been a source of some confusion. The efficacy of endothelial cell growth factor purification has been dramatically increased by the use of heparin affinity chromatography. As a result, we have been able to rapidly and efficiently purify to homogeneity 13 HBGFs by a combination of heparin affinity and reversed-phase high performance liquid chromatography. A number of their chromatographic and electrophoretic properties have been compared, and the amino acid compositions of 10 HBGFs have also been determined. We conclude that every endothelial cell growth factor described so far belongs to one of two classes, designated class 1 or class 2.

Class 1 HBGFs are anionic (pI values of about 5) and elute from heparin-Sepharose with about 1 M NaCl and from C3 reversed-phase HPLC columns with 38–42% acetonitrile. They have molecular weights of about 16,000–17,000. The

TABLE III
Amino acid compositions of class 2 HBGFs

24-h hydrolysates of 5–15 pmol of protein are shown (average of four to six independent analyses, except human brain-, human hepatoma-, and rat chondrosarcoma-derived HBGFs which are single analyses). The nearest integer values are given in parentheses.

Amino acid	Pituitary FGF ^a	Bovine brain	Bovine hypothalamus ^b	Human brain	Human cartilage	Rat chondrosarcoma	Human hepatoma
Asx	11	14.2 (14)	13.5 (14)	12.7 (13)	12.4 (12)	13.0 (13)	13.1 (13)
Glx	13	13.6 (14)	14.8 (15)	13.3 (13)	13.2 (13)	14.6 (15)	13.9 (14)
Ser	9	9.8 (10)	10.4 (10)	10.1 (10)	10.6 (11)	13.2 (13)	10.8 (11)
Gly	15	15.5 (16)	18.9 (19)	17.7 (18)	18.6 (19)	21.9 (22)	16.7 (17)
His	2	2.7 (3)	3.3 (3)	2.9 (3)	3.1 (3)	3.4 (3)	2.6 (3)
Arg	11	11.0 (11)	10.8 (11)	11.1 (11)	11.6 (12)	11.4 (11)	11.0 (11)
Thr	4	4.5 (5)	5.1 (5)	5.3 (5)	7.7 (8)	5.9 (6)	6.9 (7)
Ala	9	9.0 (9)	9.4 (9)	9.0 (9)	11.5 (12)	10.0 (10)	10.0 (10)
Pro	9	10.0 (10)	11.8 (12)	9.2 (9)	10.7 (11)	9.2 (9)	10.0 (10)
Tyr	6	6.6 (7)	6.5 (7)	6.3 (6)	7.0 (7)	7.0 (7)	6.4 (6)
Val	5	5.7 (6)	5.9 (6)	6.2 (6)	6.1 (6)	7.1 (7)	6.0 (6)
Met	2	2.1 (2)	2.5 (3)	1.6 (2)	2.3 (2)	3.4 (3)	2.7 (3)
Ile	3	3.7 (4)	3.2 (3)	4.1 (4)	4.9 (5)	3.4 (3)	4.2 (4)
Leu	12	12.1 (12)	13.4 (13)	13.4 (13)	13.2 (13)	12.8 (13)	13.4 (13)
Phe	7	7.4 (7)	7.9 (8)	7.7 (8)	6.9 (7)	8.0 (8)	8.1 (8)
Lys	13	14.0 (14)	14.1 (14)	13.6 (14)	11.5 (12)	12.2 (12)	13.3 (13)
Total	131	144	152	144	153	155	149

^a From Ref. 26: Table I, Procedure I (Reversed Phase HPLC Purification). 131 total amino acids (excluding cysteine and tryptophan). Only nearest integer values are given.

^b From Ref. 14.

amino acid compositions of these growth factors indicate that they are structurally very similar. Class 1 HBGFs include anionic endothelial cell growth factors that we have isolated from bovine brain, hypothalamus, and retina and from human brain. The acidic brain FGF described by Thomas *et al.* (5) is also in this class, and the endothelial cell growth factor described by Maciag *et al.* (6, 7, 17) is likely related or identical. Thus, these six growth factors may in reality be the same polypeptide with minor structural differences characteristic of tissue of origin and species.

Class 2 HBGFs are cationic (pI values of 8–10) and elute from heparin-Sepharose with about 1.5 M NaCl and from C3 reversed-phase HPLC columns with 29–34% acetonitrile. They have molecular weights of 18,000–19,000. Within this class, HBGFs also have similar amino acid compositions. They include endothelial cell growth factors that we have purified from bovine brain, hypothalamus, and cartilage; human brain; and rat and human tumors. The amino acid composition of bovine pituitary FGF (19), a cationic mitogen (30), indicates that it is also of this class. Thus, all of these growth factors may be the same polypeptide with minor structural differences. Growth factors from this class have also been found in peritoneal macrophages (20), human placenta (14, 22), and blood cells.²

The amino acid compositions of class 1 and 2 HBGFs have characteristic differences. Most distinctive is the high leucine content and single methionine in class 1 HBGFs. Class 2 HBGFs have more methionine and arginine, but less histidine and leucine, than class 1 HBGFs. The detailed structural relationship between the two classes is unclear. However, the NH₂-terminal sequence of class 1 anionic bovine and human brain HBGFs has been determined,³ and is distinct from the NH₂-terminus of pituitary and brain FGF (19, 26). Nevertheless, it cannot be ruled out at the present time that class 1 and 2 HBGFs might have some common domains. In particular, it would be attractive to hypothesize that they may have

a similar heparin-binding domain. Detailed amino acid sequence analysis will be required to determine how closely related HBGFs are both within a given class and between the two classes. Although it is clear that the mitogens within a given class are structurally very similar, even minor differences in sequence can have profound effects on function. For example, the GTPase activity of the p21 product of the Ha-ras oncogene is greatly reduced by a single amino acid substitution (31).

It is interesting that the three tumor-derived mitogens that we have analyzed are all class 2 HBGFs, structurally related to HBGFs found in normal tissue such as pituitary and cartilage. Both pituitary FGF (19) and cartilage-derived growth factor (32) are angiogenic, as is the chondrosarcoma-derived endothelial cell growth factor (11, 33). Thus, we speculate that tumor-induced angiogenesis (34, 35) might result in part from the abnormal production of class 2 HBGFs which themselves are normal proteins. It may be that, in such tumor-induced angiogenesis, a normal endothelial cell growth factor may be expressed at the wrong time, in the wrong place, and in inappropriate amounts.

The structural criteria reported here provide a basis for the evaluation and classification of HBGFs. As new endothelial cell growth factors are discovered, it will be of interest to determine if they are class 1 or 2 HBGFs or whether yet other classes exist.

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² M. Klagsbrun and R. R. Lobb, unpublished data.

³ D. J. Strydom and R. R. Lobb, unpublished data.

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